THE DEVELOPMENTAL POTENTIALITY OF THE LIVER-RNA-TREATED POSTERIOR PRIMITIVE STREAK IN THE CHICK EMBRYO[1]

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Previous experiments have shown that RNA-treated posterior third of the primitive streak, here abbreviated to TPS, was capable of developing into highly organized kinds of tissue. The type of tissue produced varied according to the kind of RNA used (Sanyal and Niu, 1966). Brain RNA induced the formation of neural tissue. Kidney and heart RNAs seldom induced neural formation but caused the TPS to self-differentiate into tubular and vesicular structures, respectively. On the other hand, liver (L) RNA not only induced epiblasts to become neural tissue but also made the TPS self-develop into some organized but as yet unidentifiable tissue. The presence of the latter tissue indicated the need for prolonged cultivation. In this respect, the technique (New, 1955) used was limited. In the experiments now to be reported the L-RNA-treated TPS was implanted into the coelom of 2–2 ½-day chick embryo (Hara, 1961). The choice of intracoelomic grafting was made on three grounds: (1) the implant would have the time needed to develop its acquired capability, (2) the implant would be in contact with host's incompetent cells and thus would only undergo self-differentiation, and (3) the implant would be made to the host at an early stage when visceral organs, particularly endodermal derivatives, had not yet appeared. Both L-RNA and L-protein (serum albumin, abbreviated S.A.) were employed to treat the TPS. The aim of this paper is to present data showing that: (a) S.A. has no effect on the development of TPS, (b) L-RNA stimulates both growth and differentiation, and (c) RNase-treated L-RNA reduced gut formation but promotes growth of the implants and development of feather buds even better than RNA.

Materials and Methods

Preparation of TPS

Fertilized eggs of White Leghorn chickens were obtained from Shaw Hatchery, West Chester, Pa. They were incubated for 15–18 hours at 38° C. in a forced draft incubator. The primitive streak of the blastoderm, 1.2–1.6 mm. in length and 0.2 mm. in width, was excised and stretched on agar dishes containing modified

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Locke solution (a mixture of 100 ml. NaCl (9.43 g./l.), 3.7 ml. KCL (1.2 g./100 ml.), 2.1 ml. $CaCl_2$ (2.36 g./100 ml) and 1 ml. glucose (0.2 g./100 ml.). The posterior third, 0.4–0.5 mm., was cut off and immediately transferred to the Locke solution. Accumulated pieces were divided into groups and transferred to the Locke solution (2 ml.) with or without S.A. (4 mg./ml.), L-RNA ($O.D._{260\ m\mu}$ 80/ml.) or RNase-treated L-RNA (1 mg. RNase in 2 ml. with O.D. 160). They were kept in a cold room (2–4° C.) with an occasional stir for 15–18 hours (overnight). Bovine S.A. (fraction V) was obtained from Armour and pancreatic RNase through Worthington.

Implantation of TPS

Both untreated (control) and treated TPS were implanted into the coelomic cavity of 2–2 $\frac{1}{2}$-day-old chick embryos according to the procedure of Hara with a minor modification, namely, when the host was slightly older, the graft was implanted into the posterior rather than anterior end of the cavity. This modification was made for the sole purpose of increasing the number of the recipient embryos. There was no difference in the rate of recovery, growth and differentiation between the anterior and posterior grafts. All implants were harvested at the 7th–9th day and fixed in Bouin's solution. Sections were 8 μ thick and stained with hematoxylin and eosin.

Preparation of RNA

RNA was isolated from calf liver. Thanks to Messrs. Collis and Owen of Cross Brothers Meat Packing Co., the liver was quickly removed from the slaughtered calf, sliced and put in ice-cold sucrose solution (0.25 M + $CaCl_2$, 0.003 M). The procedure of isolation has been published elsewhere (Hillman and Niu, 1963).

The glycogen of the RNA preparation was removed by Spinco centrifugation (30,000 RPM for 30 minutes) and the phenol extracted by repeated ether (or ethanol) washings, or by bubbling N_2 through. The UV absorption spectrum of L-RNA was typical of nucleic acids with maximal absorption at 260 mμ and minimal 230. The ratio of A_{260}/A_{230} exceeded 2 and that of 280/260 varied between 0.45 and 0.5. Contamination of protein, DNA and polysaccharides was routinely estimated. The amount was less than 1%. The test with orcinol yielded a green color. When a preparation did not meet the criteria given here, it would not be used for experimentation.

For functional studies of RNA, it is of prime importance to isolate the RNA within the shortest time and to use it as soon as possible. Lyophilization and storage for longer periods in a deep freezer resulted in the loss of activity except the property pertaining to growth.

Digestion of L-RNA was carried out in 2 ml. of Locke solution containing RNA and 1 mg. of RNase. The mixture was incubated at 37° C. for 1 hour. This resulted in an increase of O.D. by 35%. To an aliquot of the sample, an equal volume of ice-cold 4% perchloric acid was added. The acid-precipitable material was the undigested portion of RNA. It amounted to 15–20% of the original.

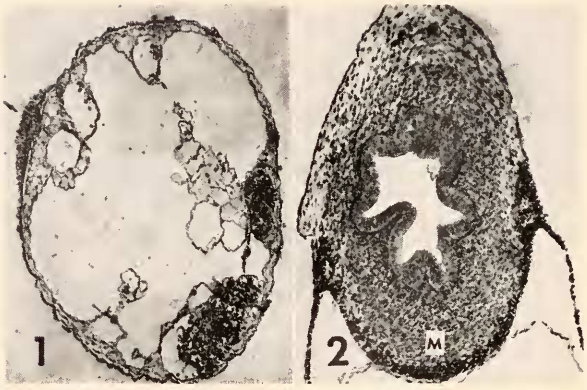


FIGURE 1. A section through the implant of control. Note the presence of endodermal epithelial lining on the outer surface (at 10:00 o'clock) and two islands of blood cells on the lower left. × 120.

FIGURE 2. A section through the L-RNA-treated implant showing intestinal vesicle. M, circular muscle. × 120.

RESULTS

Development of the Untreated TPS (controls)

A total of 60 fragments was excised. After being kept in the modified Locke solution at 2–4° C. for 15–18 hours, they were implanted into the coelomic cavity. The explants were loose and fell readily into tiny pieces, thus causing some difficulty during the process of implantation. Histological examination revealed, however, that cells of the central mass were normal and those in the outermost layer became swollen or even broken. Of the 60 controls, 23 (38%) were recovered.

The implants are either free or attached to the host tissue. Histological examination of the controls showed that the structures produced were limited to tissue with or without epidermal and/or endodermal epithelial lining. There was no appreciable difference between the untreated (control) and the S.A.-treated series, thus showing that the slight damage to the outermost layer of cells, caused by keeping the explants overnight in the cold room, had no adverse effect on their developmental potentiality. To further demonstrate this point,

TABLE 1

Developmental potentiality of the control posterior primitive streaks

Conditions of explant	Growth		Differentiation				
	Attachment to host	Size increased 3 or more times	Total	Epidermal	Epidermal-feather buds	Endodermal	Gut
Fresh controls (57)	14	3	7	5	1	5	0
Aged controls (60)	14	6	9	7	3	7	1

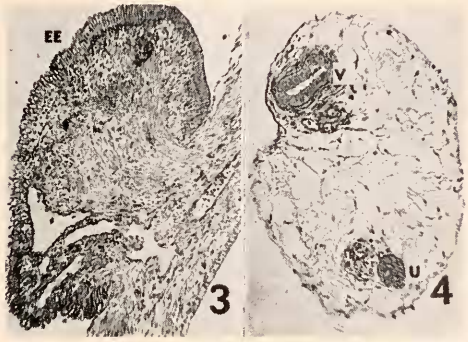


FIGURE 3. A section through a tiny portion of a big skin vesicle, developed from a L-RNA-treated TPS. The attached mass of connective tissue (see also Fig. 7) is covered by a distinctive layer of endodermal epithelium, EE. × 100.

FIGURE 4. A section through a poorly developed vesicle of the L-RNA-treated implants. V, intestinal vesicle and U, unidentified tissue. × 100.

10 anterior primitive streaks including Hensen's node were kept in the cold room for 15–18 hours. The explants showed similar loose consistency as noted in the TPS. All developed into large or very large grafts containing skin, brain, intestine, cartilage, etc.

Usually the implants were small but compact. When somewhat larger, they contained some connective tissue, blood (Fig. 1) and occasionally smooth muscle fibers. In cases when they were attached to the host, they were covered with poorly organized, vacuolated, spherical, or more or less elongated cells. These cells formed a definite layer in some cases and had the appearance of intestinal epithelium (arrow in Fig. 1).

TABLE II

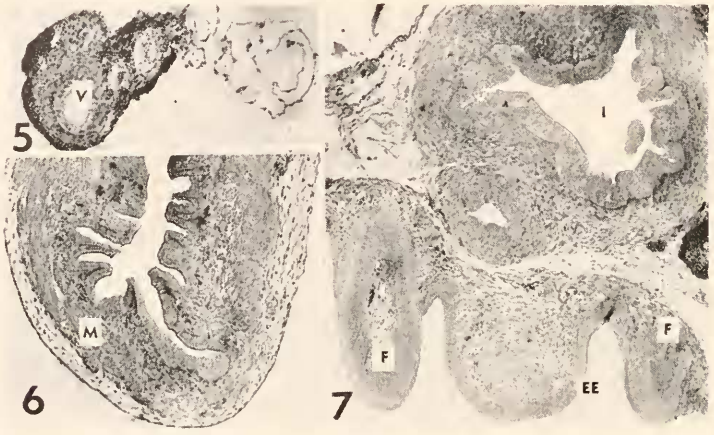
Growth and differentiation of the posterior third primitive streak treated with and without liver protein (S.A.) RNA and its hydrolytic products (A = total number, B = percentage of the total & C = percentage of the recovered)

Experimental series	Untreated (control)			Treated with S.A.			Treated with RNA			Treated with RNase-digested RNA		
	A	B	C	A	B	C	A	B	C	A	B	C
1. Implants	60	—	—	33	—	—	46	—	—	29	—	—
2. Recovered	23	38	—	13	39	—	27	59	—	23	79	—
3. Attached to host tissue	14	23	61	8	24	62	21	46	76	12	41	52
4. Size increased 3 or more times	6	10	26	2	6	15	16	35	59	14	48	61
5. Differentiation	9	15	39	6	18	46	20	43	74	14	48	56
6. Epidermal	7	12	30	3	9	23	14	30	52	9	31	39
7. Epidermal with feather buds	3	5	13	2	6	15	9	20	33	9	31	39
8. Endodermal	7	12	30	5	15	29	17	37	63	7	24	30
9. Gut	1	2	4	2	6	15	9	20	33	2	7	9

TPS were also implanted immediately into the coelomic cavity after extirpation. Of the 57 fresh controls, 37 (65%) were recovered which is superior to the 38% of the aged controls mentioned above. However, when the recovered implants were examined for growth and differentiation, the aged controls were slightly better than the fresh controls (see Table I). Although the reason for this improvement is not known, the observation undoubtedly adds support to the contention that slight damage to the explants had no adverse effect on their developmental potentiality.

Development of the S.A.-treated TPS

Thirty-three explants were kept in Locke solution with S.A. added. At the time of implantation, they were compact and looked healthy. However, the growth



FIGURES 5 and 6. Sections of two vesicles from the L-RNA-treated implants showing variations of intestinal structures. V, poorly developed intestine; M, circular muscle. × 100.

FIGURE 7. A section through a big skin vesicle obtained from RNase-treated RNA series. The lumen of the vesicle is toward the bottom. The attached mass has developed into distinctive intestinal tubes (I). EE, epidermal epithelium. I, intestinal tube, and F, feather follicle. × 120.

and differentiation of this series were remarkably similar to those of the control (Table II). It seems, therefore, that the developmental potentiality of the control and S.A.-treated TPS is similar and thus considered to be intrinsic.

Development of the L-RNA-treated TPS

At the time of implantation, the explants were similar to those of the S.A. series. A total of 46 implants were made and 27 (59%) were recovered. Most of them were attached to the host. More than half showed good growth. The overall differentiation was 43% of the total. About half (14) of the available grafts (27 cases) contained epidermal epithelium. Nine of the 14 appeared

in the form of large vesicles with feather buds, *i.e.* 64% of epidermal vesicles developed feather buds. Of the 27 recovered implants, 17 developed into tissue with endodermal epithelial lining (Figs. 3–5) and 9 into intestinal vesicles (Fig. 2) or well formed intestinal tubes (Fig. 6). It should be noted that differentiation of both epidermal and endodermal epithelial cells was usually found in the same graft. These grafts were well differentiated and rather large.

Development of the TPS treated by RNase-digested L-RNA

Incubation of L-RNA with RNase resulted in a loss of 85% of RNA. The RNase-treated L-RNA (RNase, RNA and its hydrolytic products) was used to treat 29 explants. Before implantation, they were compact and healthy. Twenty-three implants were recovered, thus providing the highest rate of recovery (79%) in our experimental series. The rate of growth was also the best (Fig. 7). The overall rate of differentiation and the formation of epidermal epithelium were similar to the RNA series. However, the development of endodermal epithelium and gut particularly decreased from 37% to 24% (a loss of 35%) and from 20% to 7% (a loss of 65%), respectively.

The effect of plain RNase on TPS was also tested. Seven explants were kept in Locke solution containing RNase. None of the implants was recovered after 10 days in the peritoneal cavity of the developing chick embryos.

Discussion

The TPS used for implantation was taken from the region where epiblasts and hypoblasts merge into a mass of cells. They are destined to give rise to connective tissue, and epidermal and endodermal epithelium (Willier and Rawles, 1931a), but are capable of being induced to develop into tissues other than epithelial cells. However, when they were grafted into the developing blastoderm, earlier experiments showed that 72% of the implants could not be located at the site of implantation and the remaining 28% appeared as mesenchyme, seldom developing into a neural tube. In the foregoing experiments, the explants were grafted into the coelomic cavity. The rate of recovery was improved (38%, Table II). Among the chemically treated series, S.A. did not alter the rate of recovery (39%), nor development. L-RNA and its hydrolytic products increased the recovery rate up to 59% and 79%, respectively. The improvement was accompanied by better growth and differentiation.

The implant of control groups developed into connective tissue with or without epidermal epithelium and/or endodermal epithelial lining. The formation of ectodermal (feather buds) or endodermal derivative (gut) was rare. Therefore, the developmental potentiality of TPS was better realized in the coelomic cavity of 2 $\frac{1}{2}$-day chick embryo than with the *in vitro* technique (New, 1955). This finding is in accord with the recent observation that the type of medium used is extremely important in the study of differentiation (Spratt and Hass, 1967).

The developmental potentiality of the control and chemically treated TPS was compared categorically in terms of total implants. It can be seen in Table II that the data of the control resemble the S.A.-treated series. Both differ significantly from RNA and RNA-digest series. The latter two series stimulate

both growth and differentiation. Of particular interest is the finding that gut formation is increased by RNA, and feather bud formation can better be facilitated by the hydrolytic products than RNA itself. Feather bud is one of the ectodermal derivatives. Its frequency of development in the epidermal vesicles is 43% of the control, 66% S.A., 64% RNA and 100% RNA digest series. Furthermore the RNA digests cut down the developing rate of endodermal epithelium (from 37% to 24%) and gut (from 20% to 7%), but increase the rate of recovery (from 59% to 79%) and the number of sizable implants (from 35% to 48%). Therefore, the reduction in development of endodermal structure is related to the loss of RNA (about 85%) and the digests are responsible for the gain in feather bud formation and growth of the implants as well. The action of the latter is not specific and belongs to the same category as reported by Sengel (1964). They perhaps contribute to the enrichment of the nutrients. It may be relevant to mention here that much of the exogeneous RNA remains intact in the recipient cells (Niu, Niu and Guha, 1968) and some are being hydrolyzed which, in turn, may be responsible for the non-specific action of RNA.

Gut was found once in 60 implants of the controls and twice in 33 S.A. and 29 hydrolyzed RNA series, respectively. This low frequency could hardly be used to argue for the action of S.A. or RNA digests on gut differentiation. Instead we consider this gut-forming ability as one of the intrinsic properties of TPS. L-RNA was capable of increasing the rate of gut formation three-fold (from 6% to 20%). This amounts to 45% of the cases with differentiation.

A double check on the developmental potentiality was made by using recovered implants (see Column C of Table II). The rate of attachment (row 3) is compared with that of the growth (row 4) and differentiation (row 5) of the same series. It can be seen that the rate of growth and differentiation is notably lowered in the control and S.A. series. On the other hand, the RNA and RNA-digest series have maintained at about the same level as the attachment. The types of differentiation are derivatives of the ectoderm and endoderm. The ectodermal derivatives of the control are similar to the S.A. series (rows 6 and 7), but strikingly different from the RNA and RNA-digest series. As to the endodermal derivatives (rows 8 and 9) the rate of development is approximately the same in the control, S.A. and RNA-digest series *but* less than half of the RNA series. In other words, the RNA used in the present experiment stimulates the formation of endodermal derivative, and this function requires the intact macromolecule of RNA. The promotion of ectodermal formation by RNA is non-specific because hydrolytic products of RNA can do almost equally well.

Liver is an organ derived from the ventral diverticulum of the gut. In the chick, its development is rather complicated and requires an inducing action from the heart rudiment (Willier and Rawles, 1931b). The fact that L-RNA can indeed stimulate gut formation is a step forward in our study on the possibility of the directed formation of specific tissue (Hillman and Niu, 1963; Niu and Leikola, 1966; Sanyal and Niu, 1966). Should L-RNA carry the message of the liver (Niu, Cordova and Niu, 1961; Zimmerman, Zoller and Turba, 1963), one immediate question is the condition under which the development of gut and liver can separately be achieved. Experiments dealing with this and other related problems are in progress.

Summary

The posterior third of the primitive streak was implanted into the 2 ½-day chick embryo. Both growth and differentiation of the untreated implants were similar to those of the serum albumin series. Treatment with liver RNA, however, resulted in a significant increase in the rate of growth and differentiation. When liver RNA was incubated with RNase, the products furthered the growth and feather bud formation, but reduced the differentiation of the endodermal derivatives, especially gut.

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